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THE DIFFERENTIATION AND CHARACTERIZATION OF CANINE ADENOVIRUSES 1 AND 2 THAT ARE USED FOR VACCINE PRODUCTION IN THE UNITED STATES

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The differentiation and characterization of canine adenoviruses 1 and 2 that are used for vaccine production in the United States

by

Cecelia Anne Whetstone

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Interdepartmental Program: Immunobiology
Major: Immunobiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Graduate College

Iowa State University

Ames, Iowa
1983

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GENERAL INTRODUCTION

Canine adenovirus type 1 (CAV-1) was isolated by Cabasso et al. in 1954 from a dog with acute hepatitis and shown to be identical to the infectious canine hepatitis (ICH) virus described by Rubarth in 1947 (7, 24). In 1962, Ditchfield et al. isolated a different canine adenovirus from throat swabs of a dog with laryngotracheitis. This strain of canine adenovirus type 2 (CAV-2) was called Toronto A26/61 (12). Several strains of CAV-1 and CAV-2 have since been described, and differentiation between the two has been based on studies of morphology (18), cytopathology in cultured cells (1, 29), serology (4, 11, 26), and pathogenicity (2, 4, 27).

Morphology, serology, and in vitro cytopathology studies have left ambiguities as to the distinctness of the two viruses. The greatest difference shown between type 1 and 2 has been in the pathogenicity of the two viruses. With CAV-1, the classical disease is infectious hepatitis, but infections can range from inapparent to fulminating fatal disease, including respiratory syndromes similar to those associated with CAV-2 infection. The virus has an affinity for hepatic parenchymal, Kupffer's, and endothelial cells. Virus has been isolated readily from the liver, kidneys, and lymphoid organs. The viremia resulting from infection with CAV-1 also leads to a phenomenon called "blue eye", a type III hypersensitivity reaction in which immune complex formation resulting from the release of virus brings about corneal endothelial damage and edema. The blue eye syndrome is attributable to both natural infection

with CAV-1 and to vaccination with modified live type 1 virus (10). The pathogenesis of CAV-2, on the other hand, is largely confined to the respiratory system, and is associated with a condition often referred to as "kennel cough". The type 2 virus does not readily cause viremia, and blue eye is not observed. Because of the potential problem of blue eye associated with vaccination with CAV-1, many veterinary biologics firms now market CAV-2 vaccines since a vaccine produced from either virus will cross-immunize against the heterologous virus (3, 8, 9, 13).

The CAVs currently used for vaccine production in the United States include the Cornell-1 and Lederle 255 strains of CAV-1 and the Toronto A26/61 (Ditchfield) and Manhattan strains of CAV-2. Until now, identification and classification of the master seed virus (the virus from which subsequent vaccine is produced) have had to come from studies on pathogenicity, morphology, cytopathology, and serology. Although taken collectively, these data may provide an accurate evaluation of the identity of a CAV type, there is currently no individual test, especially in vitro, that can do this. Additionally, although differences have been shown between CAV-1 and CAV-2 in pathogenicity (12, 27), morphology (CAV-1 fiber is 25-27 nm as compared with CAV-2 fiber which is 35-37 nm) (18), and antigenicity (11, 26,), these two viruses are still not recognized, by definition, as two distinct adenovirus species (types) (19, 34). Quantitative cross-neutralization studies with CAV-1 and CAV-2 have not shown immunologic distinctiveness by demonstrating either no cross-reaction or homologous-to-heterologous titer ratios of >16 in both directions.

The purpose of this investigation was to develop a method(s) that would differentiate, in vitro, CAV-1 from CAV-2. Additionally, it would be useful if such a method or methods could identify strains within a given type so that vaccine and field strains could be discriminated. Inherent to the success of this enterprise was the proof that CAV-1 and CAV-2 are immunologically distinct species of CAV. The experiments that were performed to accomplish these goals are described in the two papers entitled, "Restriction enzyme analysis of canine adenoviruses 1 and 2: differentiation of strains used for vaccine production in the United States," and "Characterization of the canine adenoviruses 1 and 2 by immunofluorescence, virus neutralization, and immunoprecipitation using monoclonal antibodies," which comprise the body of this dissertation.

DISSERTATION FORMAT

This dissertation is presented as an alternate format which includes two manuscripts to be submitted to scientific journals for publication. Both will be submitted to the Journal of Virology. The manuscripts are presented in the format required for the dissertation. References are cited at the end of each manuscript and are in compliance with the journal. The manuscripts are preceded by a general introduction. A summary and discussion section follows the last manuscript. Literature cited in those sections is listed after the summary and discussion.

The Ph.D. candidate, Cecelia Anne Whetstone, was the principal investigator for each of the investigations and is the sole author for both manuscripts. Dr. P. M. Gough served as major professor.

PART I: RESTRICTION ENZYME ANALYSIS OF CANINE ADENOVIRUSES 1 AND 2: DIFFERENTIATION OF STRAINS USED FOR VACCINE PRODUCTION IN THE UNITED STATES

This manuscript has been submitted for publication to the Journal of Virology.

ABSTRACT

Four strains of canine adenovirus type 1 [CAV-1 (Utrecht, Lederle 255, Cornell-1-66, Cornell-1-PK)] and two strains of canine adenovirus type 2 [CAV-2 (Manhattan, Toronto A26/61, also referred to as Ditchfield)] were examined by restriction enzyme analysis. Each of these, except Utrecht, is used for vaccine production in the United States. The two types of adenoviruses could be readily differentiated on the basis of restriction patterns obtained using either enzymes Bam Hl, Eco RI, Kpn I or Bgl II. Differences between strains within the same virus type, however, were not as conspicuous. Among the CAV-1 strains, only Cornell-1-PK showed restriction patterns that were distinct from the other strains. Between the CAV-2 strains, small pattern dissimilarities could be detected only with the use of the restriction enzymes Bam Hl and Eco Rl.

INTRODUCTION

Two types of canine adenoviruses (CAV) have been described. In 1954, Cabasso et al. isolated canine adenovirus type 1 (CAV-1) from a dog with acute hepatitis and showed this virus to be identical to the one designated by Rubarth (1947) as infectious canine hepatitis virus (9, 31). Subsequently, Ditchfield et al. (1962) isolated canine adenovirus type 2 (CAV-2) from throat swabs of a dog with laryngotracheitis (17). Although several isolates of CAV have since been described, all have been identified by serology, pathology, clinical signs, and virus morphology as either CAV-1 or CAV-2 (3, 6, 9, 15, 16, 17, 35, 37, 38, 39, 44). Although differences between the two types occur in morphology, pathogenicity, and antigenicity (1, 3, 23, 37, 38), almost all mammalian adenoviruses share group-specific determinants located on the major capsid protein, hexon (10). Because of the shared group-specific determinants, serologic responses in animals exposed to one type of CAV are cross-reactive with the other type (3, 37, 38). Moreover, immunity to either pathogen can be induced by vaccination with homologous or heterologous CAV type (5, 11, 12, 13, 19, 40). Two strains of CAV-1 (Cornell-1 and Lederle 255) and two strains of CAV-2 (Toronto A26/61 and Manhattan) are currently used for the production of vaccine in the United States. Conventional *in vitro* detection of these viruses with assays such as serum neutralization (SN) or hemagglutination inhibition are somewhat effective albeit indirect procedures for

distinguishing CAV-1 from CAV-2. None of the above techniques can be utilized for strain identification or differentiation.

Discrimination between types and even subtypes of human adenoviruses has been accomplished with the use of restriction enzyme analysis of the viral genomes (2, 26, 32, 41, 42, 43). In this study, therefore, anticipating that this technique would also be useful in differentiating CAV types and possibly strains of the same type, I examined the prototype of CAV-1 and strains of each type that are used for vaccine production in the United States. By using the restriction endonucleases Bam HI, Eco RI, Kpn I, and Bgl II, I showed that it is possible to differentiate CAV-1 from CAV-2. Furthermore, in some instances, strains of the same type demonstrated similar but unique restriction profiles characteristic of that species.

MATERIALS AND METHODS

Virus and Cells. Primary dog kidney and Madin and Darby canine kidney (MDCK) cells were maintained in Eagle's Minimum Essential Medium (MEM) with Earle's salts, 2 mM L-glutamine, 10% heat-inactivated bovine fetal serum (BFS), and gentamicin sulfate (50 mg/liter). The Utrecht and Lederle 255 strains of CAV-1 and the Toronto A26/61 (Ditchfield) strain of CAV-2 were obtained from the American Type Culture Collection, passaged one time in primary dog kidney cells, and stored at -60 C. The Cornell-1-66 and Cornell-1-PK strains of CAV-1 and the Manhattan strain of CAV-2 were a gift from Dr. L. E. Carmichael of Cornell University. As received, the Cornell-1-66 strain, a dog isolate (4), was at the third passage in primary canine kidney cells; the Cornell-1-PK strain was the 16th passage of Cornell-1-66 in primary dog kidney cell cultures; and the Manhattan strain was the fifth dog kidney passage. All three of these viruses were passaged once in primary dog kidney cells and stored at -60 C.

Virus Purification from Cell Culture. The MDCK cells were seeded at a concentration of 2×10^5 cells/ml into 850 cm^2 roller bottles containing MEM, supplemented as described. After 48 hours, 2 ml of each virus strain, diluted in MEM, were inoculated at a multiplicity of infection (MOI) of 0.2 to 0.02 onto two roller bottles and allowed to adsorb for one hour. Supplemented MEM was replaced and cells were incubated at 37 C until the CPE was 100% (about 4 to 5 days post-inoculation). Rollers were scraped to dislodge cells and the culture medium was clarified at

250 X g for 10 minutes. The supernatant fluid was then centrifuged through 40% sucrose (w/v in 0.2 M phosphate buffer, pH 7.5) at 100,000 X g for 1 1/2 hours and the resulting viral pellet was resuspended in 2.0 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6). A small volume (0.1 ml) was removed from each sample, inoculated onto MDCK cells, and examined by the FA technique for the presence of viable virus.

Viral DNA Extraction. The 2.0 ml viral pellet suspension was treated with 20 µl sodium dodecyl sulfate [(SDS) 20%], 200 µl proteinase K (1 mg/ml), and incubated at 37 C for 1 to 2 hours until cleared. Extraction was performed using an equal volume of TE buffer-saturated phenol (distilled) one time, 1/2 volumes each phenol and chloroform one time, and an equal volume of chloroform two times, taking care to save the interface until the last extraction. The DNA was then ethanol precipitated, resuspended in TE buffer, and stored either at -20 C or at 4 C until used.

Restriction Enzyme Analysis. Restriction enzymes [Bethesda Research Laboratories, Inc. (BRL)] were stored, diluted, and used in running buffers as prescribed by the company. Human adenovirus 2 (Ad 2) DNA (from BRL) was used as a standard. Enzyme reactions were stopped with 10 µl of a mixture of 0.02% bromphenol blue in 60% sucrose [w/v in Loening's buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.7)]. Cleaved DNAs were electrophoresed in a horizontal chamber at a constant 45 volts for 16 to 17 hours in a 0.8% gel (SeaKEM ME agarose) in Loening's buffer. The DNA was then stained with ethidium bromide (1 µg/ml), viewed with UV illumination of approximately 360 nm, and

photographed through a red-orange filter. Molecular weights were estimated graphically using Ad 2 as the molecular weight standard and plotting the logarithm of the fragment size against the mobility.

RESULTS

Selection and Purification of Viruses. The virus strains examined and the rationale for selecting those strains are presented in Table 1. After virus titers were determined in MDCK cells, a low MOI was used to culture viruses for DNA extraction so that the frequency of adenovirus recombination and defective interference could be held to a minimum (18). Viral CPE was 100% by days 4 to 5 post-inoculation, at which time cells were harvested. Most of the cellular debris was removed by the clarification step eliminating contamination with host cell DNA during the extraction procedure. The presence of virus in each pellet was confirmed by the FA test on MDCK cells inoculated with a small volume of the resuspended pellet.

Extraction of Viral DNA. Samples were treated with SDS and proteinase K to denature and degrade contaminating proteins. If one treatment with these agents did not yield a translucent sample, the treatment was repeated until the sample was clear. Because of the hydrophobic nature of the reaction of phenol with proteins, DNA can get trapped at the interface during extraction procedures. This problem is solved by re-extraction. Therefore, care was taken not to discard the interface until after the last extraction, which rendered noticeably better yields of DNA than when this precaution was not taken.

Differentiation of Canine Adenoviruses by Restriction Enzyme Analysis. The restriction endonucleases Bam HI, Eco RI, Kpn I, and Bgl II were chosen for this study. Each of these enzymes recognizes a

different six base pair sequence in DNA, and all have been used to map the Ad 2 genome (28), the size marker used in this study. An 0.8% gel was used because it resolves fragments from approximately 300 to 20,000 bp (33). Results of the restriction analysis are presented in Fig. 1. All enzymes cut Ad 2 DNA as predicted (28). With all enzymes used, CAV-1 and CAV-2 were easily differentiated. Among the CAV-1 strains, there was no difference between the prototype virus, Utrecht, and the Lederle 255 and Cornell-1-66 strains. For the Cornell-1-PK strain, however, the restriction enzymes cut differently than for the other CAV-1 strains in all cases. Between the CAV-2 strains, there were no pattern differences using the enzymes Kpn I or Bgl II. With Bam HI, the Toronto A26/61 strain displayed an additional submolar band just below the 14 kb fragment, and with Eco RI, this same strain showed a small fragment just above the 21 kb fragment. Neither fragment was present in the restriction patterns for the Manhattan strain. Although the Eco RI pattern for Toronto A26/61 appeared to be a partial digest, the anomally was repeatable. Genome size for all strains of CAV-1 and CAV-2 tested were estimated from the gels at 20 to 21 x 10^6 daltons, which agreed with the expected range for adenovirus (22).

DISCUSSION

Differentiation of CAV-1 from CAV-2 has previously been based on studies of pathogenicity (3, 38), serology (6, 15, 37), cytopathology in cultured cells (1), and morphology (23). Studies of cytopathology in cultured cells have not readily differentiated CAV-1 from CAV-2, and morphological studies differentiated the two viral types only on the basis of fiber length, which is 25 to 27 nm for CAV-1 and 35 to 37 nm for CAV-2 (22). Among the CAVs, the use of serology for type distinction and strain identification has not been completely successful, because the cross-reactivity of the group specific hexon, the major antigenic protein for both viruses, masks any differences that might be present in other antigenic proteins.

The greatest difference seen between type 1 and 2 has been shown in the pathogenicity of the two viruses. With CAV-1, the classical disease is infectious hepatitis, but infections can range from unapparent to fulminating fatal disease, including respiratory syndromes similar to those associated with CAV-2 infection. The virus has an affinity for hepatic parenchymal, Kupffer's, and endothelial cells. Virus has been isolated readily from the liver, kidneys, and lymphoid organs. The viremia resulting from infection with CAV-1 also leads to a phenomenon called "blue eye", a type III hypersensitivity reaction in which immune complex formation resulting from the release of virus brings about corneal endothelial damage and edema. The blue eye syndrome is attributable to both natural infection with CAV-1 and to vaccination with

modified live type 1 virus (14). The pathogenesis of CAV-2, on the other hand, is largely confined to the respiratory system, and is associated with a condition often referred to as "kennel cough". The type 2 virus does not readily cause viremia, and blue eye is not observed. Because of the potential problem of blue eye associated with vaccination with CAV-1, many veterinary biologics firms now market CAV-2 vaccines because a vaccine produced from either virus will cross-immunize against the heterologous virus (5, 11, 13, 19). Until now, identification and classification of the master seed virus (the virus from which subsequent vaccine is produced) have had to come from studies on pathogenicity, morphology, cytopathology, and serology. Restriction enzyme analysis provides a relatively simple, direct, objective, and definitive method for typing the master seed virus.

An advantage of restriction enzyme analysis over older serologic classifications is sensitivity. Serologic methods rely on distinct antigenic determinants that induce antibodies. The gene products carrying these antigenic determinants, however, represent only a few percent of the total viral genome, meaning that analysis of cross-reactions measured by serologic techniques gives information on only a few gene products, and is not necessarily representative of the relatedness of viral genomes. Sequence-specific endonucleases, such as the ones used in this study, are second only to nucleotide-sequence analysis in the detailed information that can be gained on the homologies of related genomes. This fact has been proven repeatedly with the human adenoviruses (26, 41, 42, 43); human, bovine, porcine, and equine herpes-

viruses (7, 8, 20, 21, 29, 34); parvoviruses (25); papovaviruses (36); and poxviruses (27). The restriction patterns generated with Bam HI, Eco RI, Kpn I, and Bgl II in this study all show that there are clearly two distinct genome types of canine adenovirus that can be readily differentiated from each other. Furthermore, strains of a given type that are truly different from the prototype can be detected. This is best illustrated with the Cornell-1 strains of CAV-1. The Cornell-1-66 strain, a virulent isolate from a dog suffering infectious canine hepatitis (ICH), was lyophilized after three passages in primary dog kidney cells: its restriction patterns are identical to Utrecht, the prototype virus. The Cornell-1-PK strain is an attenuated version of the same virus after 16 passages in porcine kidney cells. Its restriction patterns indicate that although it is still a type 1 CAV, it is now clearly a subtype. The Lederle 255 strain appears to be identical to Utrecht. There also seem to be small genetic differences between the CAV-2 strains detectable by restricting with Bam HI and Eco RI.

Biologically, virus attenuation can be measured by reduced virulence in the host animal. Genomically, a difference between virulent and attenuated viruses has been shown with the porcine and equine herpes viruses in which the restriction enzyme patterns of vaccine strains are distinct from field isolates (21, 29, 34). Moreover, the stability of restriction patterns from a variety of viral isolates is well-documented (7, 8, 30, 36, 41). Since vaccines are limited to the number of times they can be passed, the chance of a pattern changing would be very slight. Attenuation of a virus, therefore, is not merely a product of

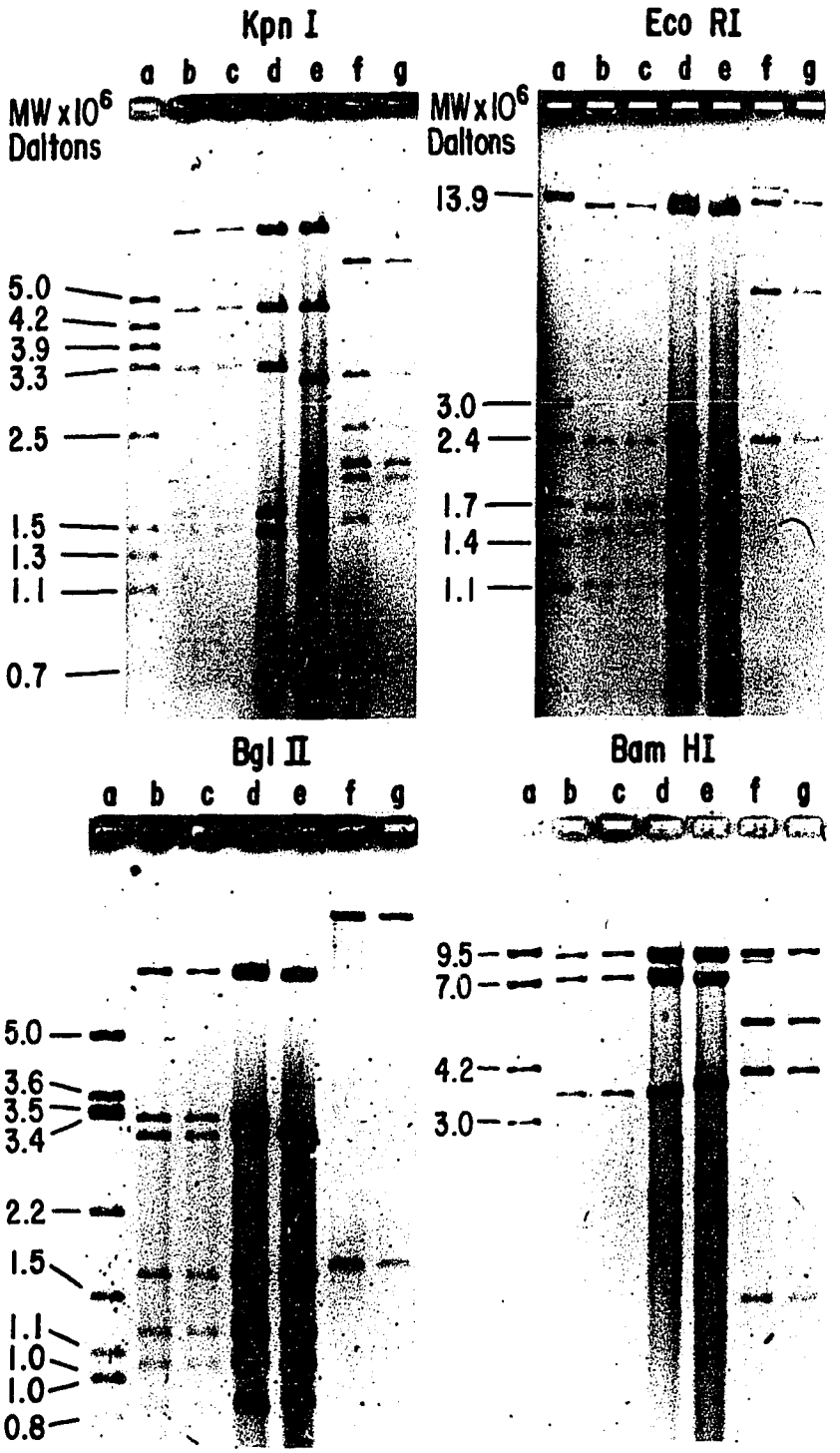
repeated passage of that virus, but rather a reflection of a genomic alteration that may be significant enough to be verified by restriction enzyme analysis. This study supports the concept that attenuated viruses, especially those that have been attenuated by passage in heterologous species cell cultures, may yield unique restriction patterns that provide a "fingerprint" of identification for that particular virus strain, a factor that becomes important in the differentiation of vaccine from field strains of virus.

Finally, according to the latest report (1982) of the International Committee on the Taxonomy of Viruses, "a species (formerly type) of adenovirus is defined on the basis of its immunologic distinctiveness, as determined by quantitative neutralization with animal antisera. If neutralization shows a certain degree of cross-reaction between two viruses in either or both directions...distinctiveness of species is assumed if...(ii) substantial biophysical/biochemical differences of the DNAs exist" (24). As already discussed, the serologic cross-reactivity of CAV-1 and CAV-2 has led to ambiguity on the definitive typing of CAV-2 as a distinct species from CAV-1. The data presented in this study indicate that there are substantial differences between these two viruses at the DNA level and that they should be recognized as separate CAV species.

TABLE 1. Virus strains used in the present study

Virus Type	Strain	Reason for inclusion into study
CAV-1	Utrecht	Prototype
CAV-1	Lederle 255	Vaccine strain
CAV-1	Cornell-1-66	(Pre)-Vaccine strain
CAV-1	Cornell-1-PK	Vaccine strain
CAV-2	Toronto A26/61	Vaccine strain
CAV-2	Manhattan	Vaccine strain

Fig. 1. Restriction enzyme analysis of CAV genomes (a) Ad2, (b) Utrecht (c) Lederle 255 (d) Cornell-1-66 (e) Cornell-1-PK (f) Toronto A26/61 (g) Manhattan.


Kpn I
a b c d e f g
MW x 10^6 Daltons
5.0
4.2
3.9
3.3
2.5
1.5
1.3
1.1
0.7
Eco RI
a b c d e f g
MW x 10^6 Daltons
13.9
3.0
2.4
1.7
1.4
1.1
Bgl II
a b c d e f g
5.0
3.6
3.5
3.4
2.2
1.5
1.1
1.0
1.0
0.8
Bam HI
a b c d e f g
9.5
7.0
4.2
3.0

ACKNOWLEDGMENTS

I thank Patricia M. Gough and Thomas O. Bunn for valuable critical review of the manuscript, and Kathleen Kelderman for preparation of the manuscript. I also extend appreciation to Leland E. Carmichael for providing seed viruses used in this study.

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PART II: CHARACTERIZATION OF THE CANINE ADENOVIRUSES 1 AND 2 BY IMMUNOFLUORESCENCE, VIRUS NEUTRALIZATION, AND IMMUNOPRECIPITATION USING MONOCLONAL ANTIBODIES

This manuscript has been submitted for publication to the Journal of Virology.

ABSTRACT

Monoclonal antibodies were produced against the Mirandola strain of canine adenovirus type 1 (CAV-1) and the Manhattan strain of canine adenovirus type 2 (CAV-2). The monoclonal antibodies were used in an indirect fluorescence-antibody (IFA) test and in an *in vitro* virus neutralization (VN) assay to examine several strains of each viral type, including those used for vaccine production in the United States. Out of 36 monoclonal antibodies produced against the Mirandola strain, 18 were type-specific for CAV-1 by IFA and 13 of those neutralized the virus *in vitro*. The other 18 antibodies bound both CAV-1 and CAV-2 by IFA; however, 7 of those specifically neutralized only CAV-1. The 160 monoclonal antibodies made against the Manhattan strain of CAV-2 yielded 77 type-specific antibodies by IFA, of which 39 neutralized only CAV-2 *in vitro*. The remaining 83 monoclonal antibodies recognized both CAV-1 and CAV-2 by IFA, with 3 of those neutralizing both viral types, and none neutralizing only CAV-2. Although type 1 CAV could be readily differentiated from type 2 CAV by using monoclonal antibodies in the IFA and VN tests, strains within each type could not be differentiated. Viral proteins were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and radioimmune precipitation (RIP) with hyperimmune dog serum to each type. Monoclonal antibodies were also used in RIP to identify the viral proteins associated with type specificity, group specificity, and neutralization.

INTRODUCTION

Two types of canine adenovirus (CAV) have been described. Canine adenovirus type 1 (CAV-1) was isolated by Cabasso et al. (1954) from a dog with acute hepatitis and was shown to be identical to the infectious canine hepatitis virus reported earlier by Rubarth (12, 43). Subsequently, Ditchfield et al. (1962) isolated canine adenovirus type 2 (CAV-2) from throat swabs of a dog with laryngotracheitis (21). In the early 1970s, several studies were done to characterize these two viruses on the basis of morphology (32), pathogenicity in vivo (3, 8, 19, 21, 50) and in vitro (1, 8, 53), and antigenicity (20, 21, 49, 50). Although some differences were demonstrated between CAV-1 and CAV-2 in all cases, the immunologic distinctness of these two viruses was not documented because of the strong cross-reactivity displayed in quantitative neutralization tests with animal sera (7, 20, 49, 50). In 1973, Tribe and Wolff showed that dogs vaccinated with hexon antigen from Toronto A26/61 CAV-2 virus were protected against virulent CAV-1 challenge (52), and the thrust of research done on these two viruses in the late 70s and early 80s centered around their immunologic relatedness in that immunity to either pathogen could be induced by vaccination with homologous or heterologous CAV types (4, 7, 14, 15, 18, 23). Consequently, two strains of CAV-1 (Cornell-1 and Lederle 255) and two strains of CAV-2 (Toronto A26/61 and Manhattan) are currently used for the production of CAV vaccine in the United States. The identification of the immunologic distinctiveness of these 2 viruses, however, has not

been pursued. Moreover, although a wealth of information has been published on the characterization of human adenoviruses both at the protein (9, 10, 11, 24, 25, 26, 34, 35, 36, 39, 40, 41, 44, 51, 54, 60) and DNA (22, 26, 40, 45, 51, 57) levels, the same has not been done for the canine adenoviruses.

My intent in this study, therefore, was twofold. The first was to characterize better the proteins of CAV-1 and CAV-2 so that a clearer understanding of the biology, immunologic relatedness, and possibly immunologic distinctiveness of the CAVs could be developed. The second was to identify any differences that might exist among strains of CAV-1 and CAV-2 so that vaccine strains could be differentiated from field strains.

To accomplish these goals, monoclonal antibodies were produced against two virulent strains of CAV, the Mirandola strain of CAV-1 and the Manhattan strain of CAV-2. These were then tested by the indirect fluorescent-antibody (IFA) technique and by virus neutralization (VN) against both the original virus from which each was prepared as well as from the heterologous virulent virus. The IFA and VN tests showed monoclonal antibodies with type as well as group specificities. Neutralization was associated with both type and group-specific antibodies. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) profiles were performed as well as radioimmune precipitation (RIP) with dog origin CAV-specific antiserum to each [^{35}S]methionine-labeled virus to identify the major structural polypeptides. Monoclonal antibodies were then used in RIP assays to identify which structural polypeptides

were precipitated by antibodies that were type-specific, group-specific, showed type or group specificity in neutralization, or were group-specific but showed type-specific neutralization.

A bank of monoclonal ascites from each fusion were also tested by IFA and VN against the Utrecht (prototype), Cornell-1-66, Cornell-1-PK, and Lederle 255 strains of CAV-1 and the Toronto A26/61 strain of CAV-2 in order to identify virus strains useful in differentiating vaccine and field virus strains. With the techniques used, however, no detectable differences between strains of the same CAV type could be found.

MATERIALS AND METHODS

Virus and Cells. Primary dog kidney and Madin and Darby canine kidney (MDCK) cells were maintained in Eagle's Minimum Essential Medium (MEM) with Earle's salts, 2 mM L-glutamine, 10% heat-inactivated bovine fetal serum (BFS), and gentamicin sulfate (50 mg/liter). The Utrecht and Lederle 255 strains of CAV-1 and the Toronto A26/61 (Ditchfield) strain of CAV-2 were obtained from the American Type Culture Collection, passaged one time in primary dog kidney cells, and stored at -60 C. The Cornell-1-66 and Cornell-1-PK strains of CAV-1 and the Manhattan strain of CAV-2 were a gift from Dr. L. E. Carmichael of Cornell University. As received, the Cornell-1-66 strain, a dog isolate (6), was at the third passage in primary canine kidney cells; the Cornell-1-PK strain was the 16th passage of the Cornell-1-66 in primary pig kidney cell cultures; and the Manhattan strain was the fifth dog kidney passage. All three of these viruses were propagated in primary dog kidney cells and stored at -60 C. The Mirandola strain of CAV-1 was from a stock culture kept at the National Veterinary Services Laboratories (NVSL). The virus was at passage level 31-35 in primary dog kidney cells and stored at -60 C. Purified virus samples were prepared by centrifuging tissue culture supernatant fluids through 40% sucrose (w/v in 0.2 M phosphate buffer, pH 7.5) at 100,000 X g for 1-1/2 hours, then band purifying the pellet in a 15-45% discontinuous sucrose gradient.

Monoclonal Antibody Production. The method used for the production of the monoclonal antibodies was the same as previously described by this

author (55). Briefly, Balb/c mice were inoculated intraperitoneally (i.p.) with 0.5 ml of purified virus from either the Mirandola strain of CAV-1 or the Manhattan strain of CAV-2. After 2-3 weeks, serum samples from the mice were tested by IFA for the presence of antibody to CAV. Three days prior to fusion, sero-positive mice were inoculated a second time intravenously with 0.2 ml of the same virus. Spleen cells from immunized mice were fused with nonsecretor sp2/0 myeloma cells and culture fluids from the resulting hybridomas were screened by IFA for the presence of antibody. Cloning was carried out in soft agar and the final monoclonal antibody products were collected as ascites fluids from pristane-primed Balb/c mice that had been injected i.p with selected clones.

IFA Test. MDCK cells were prepared (2×10^5 cells/ml) in 8-chamber tissue culture slides, incubated in an humidified atmosphere of 5% CO_2 at 37 C for 24 hours, inoculated with CAV at a multiplicity of infection (MOI) of 0.5 - 0.1, and fixed at 24 hours post-inoculation in acetone. Dilutions of 1:10, 1:100, 1:1000, and 1:10,000 of monoclonal ascites (50 µl) in phosphate-buffered saline were incubated on the cells for 30 minutes at 37 C in a humidified chamber. One well was used for each dilution. Cells were washed in phosphate-buffered saline and stained with a 1:150 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin G (IgG) (heavy and light chain specific) antibody (Cappel Laboratories) for 30 minutes. Controls included: CAV-inoculated and uninoculated MDCK cells stained by direct FA with FITC-labeled dog anti-CAV antiserum; inoculated cells stained by direct

FA with the goat anti-mouse conjugate; and uninoculated cells stained by IFA with the monoclonal antibodies.

Virus Neutralization. The ability of the monoclonal antibodies to neutralize CAV was assayed by a varying virus-constant antibody test carried out in 96-well tissue culture plates. Monoclonal ascites were diluted 1:50 or 1:100 in phosphate-buffered saline, mixed with an equal volume of virus at each dilution (10^{-1} through 10^{-8}), and inoculated (50 µl) into each of four wells containing fresh monolayers of MDCK cells in MEM supplemented with 10% BFS. Controls included uninoculated cells and cells inoculated with virus titrations (25 µl/well) without antibody. After 72 hours and at 6 days post-inoculation, cells were read for viral cytopathogenic effect (CPE) and titers were calculated by the Reed and Muensch 50% endpoint technique (42). A neutralization of $\geq$2.0 logs of virus was considered indicative of specific activity.

Radiolabeling of Virus, RIP, and Polyacrylamide Gel Electrophoresis. CAV-infected and sham-infected MDCK cells were radiolabeled with [^{35}S]methionine and analyzed by radioimmune precipitation (RIP) on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel, in accordance with a previously published method (28). MDCK cell monolayers growing in 150 cm^2 flasks were infected at a high MOI. After 19 hours, the virus and cells were labeled with 10 µCi/ml of [^{35}S]methionine in methionine-deficient medium for 4 hours. Cells were then sonicated, solubilized in cold extraction buffer (1% NP-40, 0.1 mM phenylmethylsulfanyl fluoride, pH 8.0), and clarified. Either 20 µl of dog anti-CAV serum or 5 µl of monoclonal antibody was added to labeled cell lysates and precipitated

with protein A-Sepharose CL-4B beads. Samples were dissolved in Laemmli buffer (29) containing 2-mercaptoethanol and electrophoresed on SDS-10% polyacrylamide gel with either 2.7% or 1.3% N,N'-methylene-bis-acrylamide (bis) crosslinker. A monoclonal antibody to pseudorabies virus (PRV) was used as a control and molecular size markers were run in the first and last lanes of each gel.

Non-radiolabeled viruses from cell extracts were electrophoresed on SDS-PAGE by the Laemmli method and stained with Coomassie Brilliant Blue R-250 (29).

RESULTS

Isolation and Characterization of Monoclonal Antibodies to CAV-1 and CAV-2 by IFA and VN. Thirty-six monoclonal antibodies against the Mirandola strain of CAV-1 and 160 monoclonal antibodies against the Manhattan strain of CAV-2 were produced. For screening purposes all antibodies were initially tested by IFA against only those two viruses and uninoculated MDCK cells. Hybridomas with fluids positive on the uninoculated controls were culled as secretors against tissue culture antigens. Using CAV-specific antibodies, secreted by different hybridomas, various staining patterns for CAV-infected MDCK cells were observed. Although nuclear staining was always present, it varied from large, lobular inclusions that were usually associated with the nuclear membrane, to small, pinpoint inclusions that were diffused throughout the nucleus. Some diffuse staining of the cytoplasm was also noted, usually in conjunction with all types of nuclear staining except the diffuse, pinpoint type.

After monoclonal ascites were produced, all antibodies were further tested by VN against Mirandola and Manhattan. The 1:50 or 1:100 dilution of each antibody that was used for VN was determined by its titer as calculated from the IFA test. Monoclonal antibodies with IFA titers $\geq$1:1000 were used at 1:100, and those with a lower titer were used at 1:50.

Additionally, 20 of the CAV-1 monoclonal antibodies and 34 of the CAV-2 monoclonal antibodies were assayed by IFA and VN against the

Utrecht, Lederle 255, Cornell-1-66, and Cornell-1-PK strains of CAV-1 and the Toronto A26/61 strain of CAV-2. Those virus strains were chosen because they were representative of the strains used for vaccine production in the United States or because they were a prototype virus (Utrecht). Among the strains within each type, either CAV-1 or CAV-2, there were no differences detected by either assay. However, between the two types, distinctions could be made both in the IFA and VN tests as illustrated in Table 1.

There were 18 different IFA type-specific monoclonal antibodies to CAV-1, 13 of which also neutralized the virus, and 77 type-specific monoclonal antibodies to CAV-2, 39 of which were neutralizing. Of the 101 antibodies that bound both viruses in the IFA test, 3 of those also neutralized both viruses. Those 3 monoclonal antibodies were from the CAV-2 fusion. Also, among the antibodies with specificity for both viruses by the IFA technique, there were 7 monoclonal antibodies from the CAV-1 fusion that neutralized only CAV-1.

Characterization of the Structural Proteins of CAV-1 and CAV-2. The results of SDS-PAGE analysis of whole virus are shown in Fig. 1. Based on the exhaustive characterization that has been done on the human adenoviruses (24, 26, 40, 63), tentative assignations were made for the viral proteins: hexon (II), penton (III), fiber (IV), fiber-associated protein (IVa1), hexon-associated proteins (IIIa,VI,VIII), core proteins (V,VII), and core-associated protein (IVa2). These are depicted more clearly in Fig. 2-4 in lanes showing RIP of [^{35}S]methionine-labeled

CAV with dog CAV-1 and CAV-2 specific antisera. Estimated apparent molecular weights (MW) for the major structural polypeptides were calculated by plotting the relative rate of migration for the polypeptide band against the log of the MW. These data are presented in Table 2, and show good correlation with what has been reported for analogous polypeptides from human adenoviruses (24, 26, 40, 51, 63). Although most of the structural polypeptides were precipitated in the gel in Fig. 2, the major antigenic proteins, i.e. hexon (II), penton (III), fiber (IV,IVal), and hexon-associated peripentonal protein (IIIa), were not well-resolved. In the gels shown in Fig. 3 and 4, therefore, the percent of bis cross-linker was decreased from 2.7% to 1.3% and electrophoresis was carried out for a longer period of time. The resulting gels gave good separation of the higher MW components allowing for more detailed examination of polypeptides precipitated in the RIPs.

Both homologous (Fig. 2, lane b; Fig. 3, lane b; Fig. 4, lane c) and heterologous (Fig. 2, lane c; Fig. 3, lane c) dog origin CAV type-specific antisera showed similar RIP bands to both CAV-1 and CAV-2. The only dissimilarity noted was in Fig. 3 where there appears to be less precipitation of some of the fiber and peripentonal hexon-associated polypeptides of CAV-1 by CAV-2 dog antiserum. CAV-1 dog antiserum did not precipitate any polypeptides (Fig. 4, lane b) from sham-infected MDCK cells (Fig. 4, lane a).

Representative CAV monoclonal antibodies that had shown different specificities in the IFA and VN tests were chosen for use in the RIP. Results of those precipitations are shown in Fig. 3 and 4, lanes e-h, and

presented in Table 3. In Fig. 3, [^{35}S]methionine-labeled CAV-1 was used as antigen and in Fig. 4, [^{35}S]-CAV-2 was utilized. In lane d of each gel, a PRV monoclonal antibody was used as a background control. The RIP in lane e of each gel was carried out with type-specific monoclonals (by IFA) that did not neutralize. In both cases, the monoclonal antibodies bound to an antigenic determinant located on fiber polypeptides. Additionally, the CAV-1 specific antibody detected the same antigen on hexon-associated peripentonal protein (IIIa). In lane f of each gel, where group-specific non-neutralizing monoclonal antibodies were used, hexon (II) polypeptide was precipitated in CAV-2 but fiber (IV,IVal) proteins were precipitated by CAV-1. The IV polypeptide in Fig. 3, lane f, although only faintly visible in the autoradiogram, became more evident upon longer exposure. Type-specific, neutralizing monoclonal antibodies were utilized in lane g of each gel; for each virus the same polypeptides were precipitated with both neutralizing (lanes g) and non-neutralizing (lanes e) type-specific antibodies. In Fig. 3, lane h, a group-specific monoclonal antibody that specifically neutralized CAV-1 was used. Again, fiber (IV, IVal) and hexon-associated peripentonal (IIIa) polypeptides were precipitated. With CAV-2, Fig. 4, lane h, the hexon (II) polypeptide was precipitated by a group-specific monoclonal antibody that also neutralized both viruses.

DISCUSSION

Since the early 1960s it has been believed that two types of canine adenoviruses might exist. However, because quantitative cross-neutralization studies with CAV-1 and CAV-2 could not, by definition, separate these two viruses into distinct adenovirus species (types), CAV-1 is still the only provisionally recognized species of CAV (33, 63). And yet, studies of the pathology (3, 8, 19, 21, 50), morphology (32), and antigenicity (20, 21, 49, 50) of these viruses indicate that there are indeed significant differences between the two, similar to the types of differences recognized in disparate species of human adenoviruses (48, 56, 57, 58).

It has been shown that monoclonal antibodies can be useful tools in defining antigenic determinants (17, 30, 37, 46, 59) that can lead to typing viruses (38), defining heterogeneity between serotypes and between viruses which appear to be related according to neutralization data (5, 16, 27, 28, 31), and even differentiating between wild and vaccine strains (47, 62). In order to validate the possible immunologic distinctiveness of the CAVs, therefore, I prepared a bank of monoclonal antibodies against the virulent Mirandola and Manhattan strains of CAV-1 and CAV-2 respectively. These two strains were chosen because they are the standard CAV strains used at the NVSL to challenge the efficacy of CAV vaccines licensed by the United States Department of Agriculture. I hoped that, in addition to validating species distinctness of the CAVs

through type-specific monoclonal antibodies, I also could differentiate strains and, ultimately, separate vaccine and wild strains.

Eighteen of 36 monoclonal antibodies produced against CAV-1 and 77 out of 160 monoclonal antibodies produced against CAV-2 were type-specific. This indicates the possibility that these two viruses are different. In order for the two viruses to be recognized as different species, however, several antibodies recognizing a significant number of different epitopes must be derived. Epitope mapping of the CAVs using neutralizing monoclonal antibodies remains to be done. However, fluorescence patterns shown by monoclonal antibodies in the IFA test on MDCK cells at the same stage of CAV infection, i.e. 24 hours post-inoculation, displayed a wide diversity indicating that a variety of proteins were being specifically recognized by antibodies from different hybridomas. Although this does not preclude the possibility of shared determinants between proteins, it does increase the likelihood that the antibodies are binding to different epitopes.

The CAV-neutralizing monoclonal antibodies produced in this study are significant for several reasons. Although monoclonal antibodies have been produced against human adenovirus proteins (2, 13), none has been reported to have neutralizing capabilities. These CAV antibodies then provide a tool for studies on adenovirus neutralization, including epitope mapping as well as mechanisms of neutralization. Thirteen CAV-1 and 39 CAV-2 monoclonal antibodies were type-specific, reinforcing the concept of immunologic distinctiveness between these two viruses. Moreover, given the fact that 3 out of 83 group-specific CAV-2 antibodies

and 7 out of 18 group-specific CAV-1 antibodies were also involved in neutralization, one might conclude that neutralization may be closely linked to type specificity, but that more than one antigenic site may be involved.

Prior to this study, protein analysis of the structural polypeptides of CAV by SDS-PAGE had not been reported. In the characterizations reported here, the Mirandola strain of CAV-1 and the Manhattan strain of CAV-2 were utilized so that immune precipitations could also be performed both with dog CAV strain-specific antisera and the CAV monoclonal antibodies. Structural polypeptides that correlated well with those reported for human adenoviruses (26, 40, 63) were noted in both SDS-PAGE of whole virus cell-culture extracts and in RIPs of [^{35}S]methionine-labeled CAV with dog strain-specific antisera (Table 2). The only notable difference between the two viruses was the lack of detection of the hexon-associated peripentonal (IIIa) polypeptide in CAV-2 by CAV-2 dog antiserum (Fig. 4, lane c), whereas this same antiserum did precipitate the IIIa polypeptide in CAV-1 (Fig. 3, lane c). A possible explanation for this is discussed later.

Much has been written about the immunogenic properties of adenoviruses, and models for type and group-specific binding and/or neutralization have been proposed (26, 36, 40, 51). Since a variety of binding specificities were noted for the monoclonal antibodies in the IFA and VN tests in this study (Table 1), a representative monoclonal from each specificity group was selected and tested in RIPs to identify which polypeptides would be immunoprecipitated (Table 3). With CAV-1 and CAV-2,

both neutralizing and non-neutralizing monoclonal antibodies bound to the fiber polypeptides IV and IVal (Fig. 3 and 4, lanes e and g). Additionally, however, the CAV-1 monoclonal antibodies precipitated what appears to be the hexon-associated peripentonal IIIa polypeptide. In referring back to the RIPs with dog antisera, it was noted that the IIIa polypeptide was not precipitated from CAV-2 by CAV-2 dog antiserum. From this, one can propose that there are shared antigens on fiber and IIIa polypeptide in CAV-1 and at least fiber but possibly also IIIa protein in CAV-2 that are distinct and important in type specificity and type-specific neutralization. The presence of some precipitation of IIIa polypeptide from CAV-1 by CAV-2 dog serum (Fig. 3, lane c) supports the idea of the shared antigen. Possibly, since the fiber of CAV-2 is longer than that of CAV-1, the hexon-associated peripentonal protein of CAV-2 is less accessible to the immune system and to antibodies. An RIP with CAV-2 antibody and CAV-2 antigen, therefore, would not precipitate IIIa polypeptide whereas an RIP with CAV-2 antibody and CAV-1 antigen, with shorter fiber and less steric hindrance to antibody binding, would show immunoprecipitation of IIIa polypeptide. If IIIa has a shared antigenic determinant with another protein such as fiber, the CAV-2 antiserum would contain antibody that would bind to IIIa, although probably in a lesser quantity than occurs with CAV-1 antiserum (Fig. 3, lane c).

Group-specific monoclonal antibodies from the CAV-2 fusion, both neutralizing and non-neutralizing, immunoprecipitated hexon (II) of CAV-2 (Fig. 4, lanes f and h). This supports prior evidence for the group specificity of hexon antigen which has been documented for adenoviruses

of many animal species (13, 36, 57). The interesting point here is that type-specific neutralization is associated with fiber protein and group-specific neutralization is tied to hexon protein.

With monoclonal antibodies from the CAV-1 fusion, a different pattern emerges. The group-specific antibodies immunoprecipitated the IV and IVa1 polypeptides of fiber instead of hexon (Fig. 3, lanes f and h). Moreover, the neutralizing monoclonal antibody also immunoprecipitated the IIIa polypeptide of the hexon-associated peripentonal protein. The unique feature of this antibody is that it is group-specific in binding both viruses in the IFA test, but type-specific in neutralizing only CAV-1 in the VN test. The RIP results shown in Fig. 3 suggest that the binding of the IIIa protein is important in neutralization of CAV-1. Further testing with additional monoclonal antibodies needs to be done to substantiate and perhaps further elucidate these ideas.

It is interesting to note that the group-specific neutralizing antibodies from the CAV-2 fusion all neutralize both CAV-1 and CAV-2, but that the group-specific neutralizing antibodies from the CAV-1 fusion only neutralize CAV-1. Cross-protective immunity studies with the CAVs have shown that vaccination with CAV-2 in dogs stimulates a serologic response that is completely protective against challenge with either virulent CAV-1 or virulent CAV-2 (7, 14, 15, 18). Dog vaccination with CAV-1, on the other hand, produces a serologic response that is completely protective against challenge with virulent CAV-1 but only partially protects against challenge with CAV-2, with challenge virus being recovered for a week after exposure (23). Antibodies raised

against CAV-1 that bind to the fiber and peripentonal proteins of CAV-1 and neutralize, possibly by preventing or sterically hindering attachment of the virus, might also bind to similar proteins in CAV-2. However, since the fiber of CAV-2 is longer, it would still be able to attach to cells if antibodies were directed toward an antigenic determinant(s) near the base of the fiber leaving the tip free. On the other hand, group-specific antibodies directed against hexon attach to both viruses in the same physical position facilitating neutralization in the same manner for both viruses. This supports the experimental results of Tribe and Wolff (52) who protected dogs against CAV-1 challenge by vaccinating with CAV-2 hexon.

Strain differentiation among the CAVs was attempted by testing a selected bank of the monoclonal ascites from each fusion against the Utrecht, Cornell-1-66, Cornell-1-PK, and Lederle 255 strains of CAV-1 and the Toronto A26/61 strain of CAV-2. In the IFA and VN tests, with the antibodies used, no differences could be detected between results observed for these viruses and data obtained with Mirandola and Manhattan. The possibility of using monoclonal antibodies to differentiate vaccine from field strains, therefore, does not seem likely.

The feasibility of using monoclonal antibodies to differentiate CAV-1 from CAV-2 is good. The fact that by random chance approximately half of the antibodies from each fusion was type-specific lends credence to the idea that there are indeed two species of CAV. This would especially be true if those antibodies recognized a range of different antigens. I have examined the DNA of the CAV strains used in this study

by restriction enzyme analysis and have found the genomes of type-1 virus strains to be very different from the genomes of type-2 virus strains (61). This finding in conjunction with the results from this experiment suggest that CAV-1 and CAV-2 should both be recognized as species in the genus Mastadenovirus in the family Adenoviridae.

TABLE 1. Binding specificities of CAV monoclonal antibodies

Specificity of monoclonal antibodies	Virus used to produce monoclonal antibody	
	CAV-1[a]	CAV-2[b]
Binds only one virus by IFA[c]	18/36[d]	77/160
Binds only one virus by IFA and VN[e]	13/36	39/160
Binds both viruses by IFA	18/36	83/160
Binds both viruses by IFA and VN	0/36	3/160
Binds both viruses by IFA but only one virus by VN	7/36	0/160

[a]Mirandola strain.

[b]Manhattan strain.

[c]Indirect fluorescent-antibody technique using fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G antibody (Cappel Laboratories).

[d]Designates number positive out of total number tested.

[e]Virus neutralization carried out in vitro.

Table 2. Apparent molecular weights of CAV structural polypeptides

Protein	Polypeptide	Polypeptide Molecular Weight[a]	
		CAV-1[b]	CAV-2[c]
Hexon	II	132,000	128,000
Penton	III	92,000	87,000
Fiber	IV	58,000-62,000	58,000-60,000
Fiber-associated	IVa1	56,000	57,000
Hexon-associated III (peripentonal)	IIIa	65,000	n.k.[d]
Core-associated	IVa2	50,000	53,000
Minor core	V	44,000	48,000
Hexon-associated I	VI	n.k.	27,000
Major core	VII	n.k.	25,000
Hexon-associated II	VIII	n.k.	18,700
Hexon-associaed I (groups of 9)	IX	n.k.	17,500

[a]Calculated from radioimmune precipitations with dog origin, CAV type-specific antiserum.

[b]Mirandola strain.

[c]Manhattan strain.

[d]n.k. = not known.

Table 3. Structural polypeptides of CAV immunoprecipitated by monoclonal antibodies in an RIP

Monoclonal antibody	Virus used for fusion	Specificity in IFA[a]	Specificity in VN[b]	Polypeptide bound in RIP[c]
4A1-C9	CAV-1	CAV-1	neg.	fiber (IV, IVal) hexon-associated peripentonal (IIIa)
4B9-B7	CAV-1	CAV-1 and CAV-2	neg.	fiber (IV, IVal)
2E10-G7	CAV-1	CAV-1	CAV-1	fiber (IV, IVal) hexon-associated peripentonal (IIIa)
2G5-G4	CAV-1	CAV-1 and CAV-2	CAV-1	fiber (IV, IVal) hexon-associated peripentonal (IIIa)
3G4-B10	CAV-2	CAV-2	neg.	fiber (IV, IVal)
5D6-B11	CAV-2	CAV-1 and CAV-2	neg.	hexon (II)
3B5-D9-F12	CAV-2	CAV-2	CAV-2	fiber (IV, IVal)
1C12-G8	CAV-2	CAV-1 and CAV-2	CAV-1 and CAV-2	hexon (II)

[a] IFA = Indirect fluorescent antibody technique.

[b] VN = Virus neutralization in vitro.

[c] RIP = Radioimmune precipitation.

Fig. 1. SDS-PAGE of CAV structural polypeptides. Tissue culture fluids from CAV-infected and sham-inoculated MDCK cells were clarified by low speed centrifugation, pelleted through 40% sucrose, electrophoresed on an SDS-10% polyacrylamide gel, with 2.7% bis, under reducing conditions, and stained with Coomassie Brilliant Blue R-250. Lane a. MW markers. Lane b. Control sham-inoculated cell lysate. Lane c. CAV-1, Mirandola strain. Lane d. CAV-2, Manhattan strain. Proteins identified include: hexon (II), penton (III), hexon-associated III peripentonal (IIIa), fiber (IV), fiber-associated (IVal), core-associated (IVa2), minor core (V), hexon-associated I (VI), major core (VII), hexon-associated II (VIII). MW markers are identified.

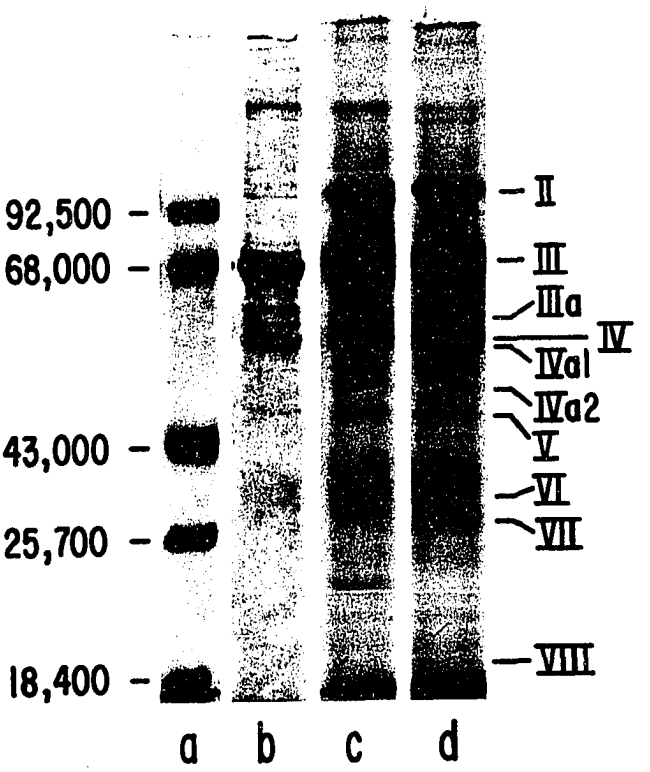
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Fig. 2. RIP of [^{35}S]methionine-labeled CAV-2 structural polypeptides. CAV-2-infected (Manhattan strain) MDCK cells were labeled with 10 μCi/ml of [^{35}S]methionine for 4 hours, detergent disrupted, and sonicated. Proteins were immunoprecipitated with 20 μl of CAV-1 or CAV-2 dog antiserum and protein A-Sepharose CL-4B beads. The immunoprecipitates were electrophoresed on an SDS-10% polyacrylamide gel, with 2.7% bis, under reducing conditions. Lane a. CAV-2-infected cell lysate. Lane b. RIP of CAV-2 by dog anti-CAV-2-Manhattan antiserum. Lane c. RIP of CAV-2 by dog anti-CAV-1-Mirandola antiserum. Proteins identified include: hexon (II), penton (III), hexon-associated III peripentonal (IIIa), fiber (IV), fiber-associated (IVa1), core-associated (IVa2), minor core (V), hexon-associated I (VI,IX), major core (VII), hexon-associated II (VIII). MW markers were identified from standards electrophoresed in this gel.

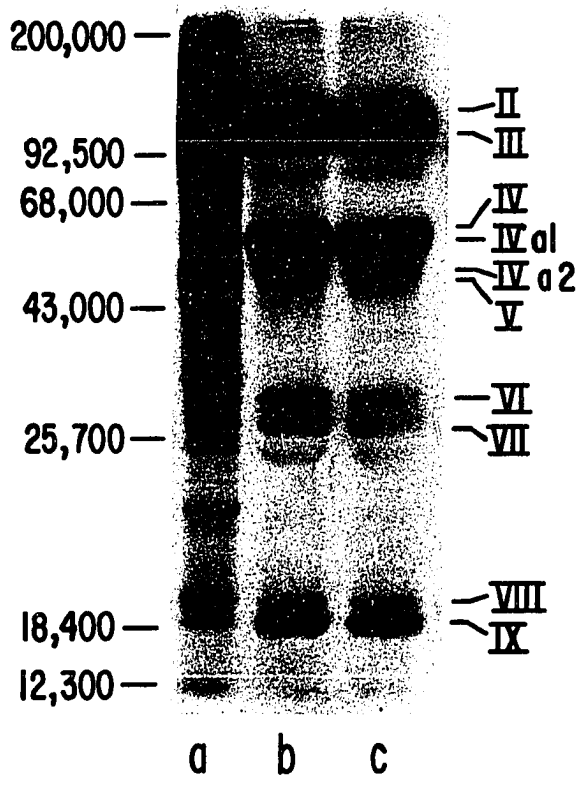
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Fig. 3. RIP of [^{35}S]methionine-labeled CAV-1 structural polypeptides. CAV-1-infected (Mirandola strain) MDCK cells were labeled with 10 μCi/ml of [^{35}S]methionine for 4 hours, detergent disrupted, and sonicated. Proteins were immunoprecipitated with either 20 μl of CAV-1 or CAV-2 dog antiserum or 5 μl of monoclonal antibody ascites and protein A-Sepharose CL-4B beads. The immunoprecipitates were electrophoresed on an SDS-10% polyacrylamide gel, with 1.3% bis, under reducing conditions. Lane a. CAV-1-infected cell lysate. Lane b. RIP of CAV-1 by dog anti-CAV-1-Mirandola antiserum. Lane c. RIP of CAV-1 by dog anti-CAV-2-Manhattan antiserum. Lane d. Control RIP of CAV-1 by PRV monoclonal antibody. RIP of CAV-1 by monoclonal antibodies 4A1-C9 (lane e), 4B9-B7 (lane f), 2E10-G7 (lane g), 2G5-G4 (lane h). Proteins identified include: hexon (II), penton (III), hexon-associated peripentonal (IIIa), fiber (IV), fiber-associated (IVa1), core-associated (IVa2), minor core (V). MW markers were identified from standards electrophoresed in this gel.

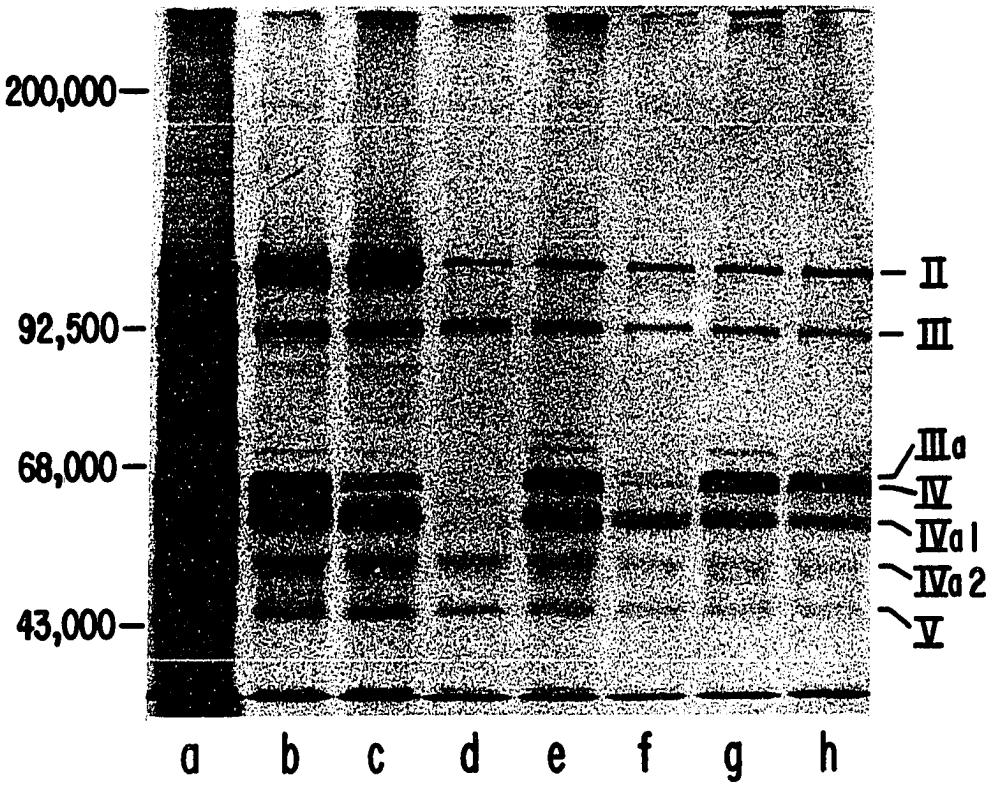
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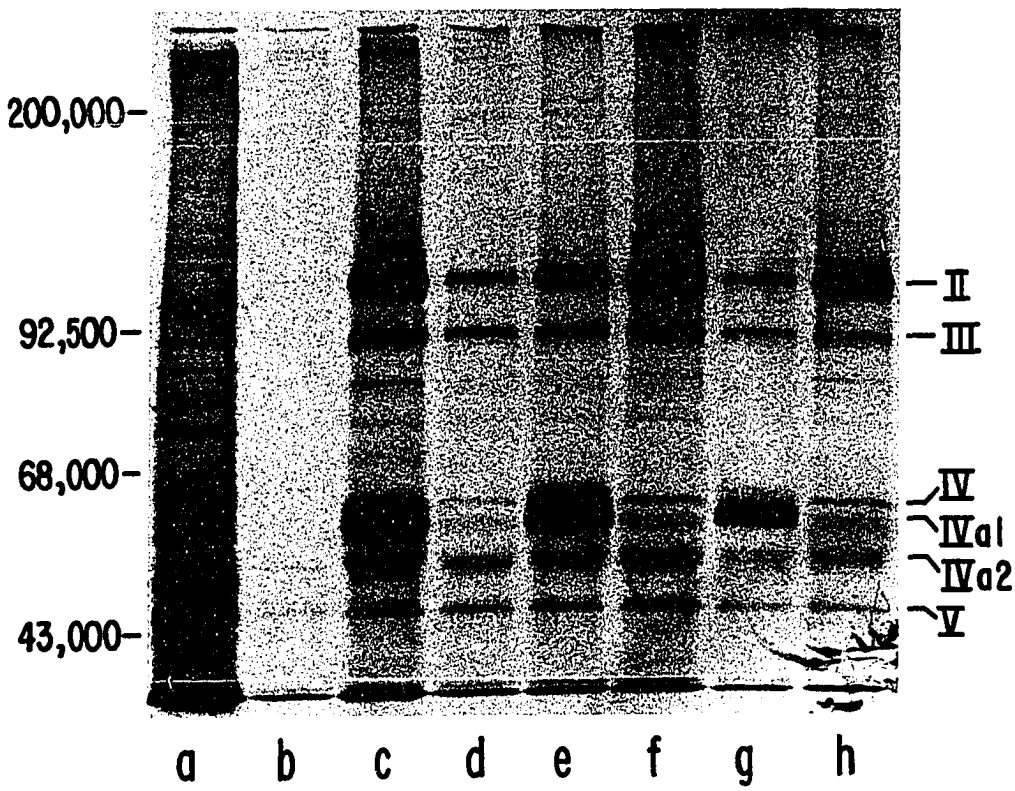
Fig. 4. RIP of [^{35}S]methionine-labeled CAV-2 structural polypeptides. CAV-2-infected (Manhattan strain) or sham-inoculated MDCK cells were labeled with 10 μCi/ml of [^{35}S]methionine for 4 hours, detergent disrupted, and sonicated. Proteins were immunoprecipitated with either 20 μl of CAV-1 or CAV-2 dog antiserum or 5 μl of monoclonal antibody ascites and protein A-Sepharose CL-4B beads. The immunoprecipitates were electrophoresed on an SDS-10% polyacrylamide gel, with 1.3% bis, under reducing conditions. Lane a. Sham-inoculated control cell lysate. Lane b. RIP of control cell lysate by dog anti-CAV-1-Mirandola antiserum. Lane c. CAV-2-infected cell lysate. Lane d. Control RIP by PRV monoclonal antibody. RIP of CAV-2 by monoclonal antibodies 3G4-B10 (lane e), 5D6-B11 (lane f), 3B5-D9-F12 (lane g), 1C12-G8 (lane h). Proteins identified include: hexon (II), penton (III), hexon-associated peripentonal (IIIa), fiber (IV), fiber-associated (IVa1), core-associated (IVa2), minor core (V). MW markers were identified from standards electrophoresed in this gel.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ACKNOWLEDGMENTS

I thank Patricia M. Gough for helpful discussions and valuable critical review of the manuscript. I extend gratitude and appreciation to Ione Peterson for dedicated and skillful technical assistance, and thank Dr. L. E. Carmichael for providing seed viruses used in this study.

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SUMMARY AND DISCUSSION

Monoclonal antibodies against the Mirandola strain of canine adenovirus type 1 (CAV-1) and the Manhattan strain of canine adenovirus type 2 (CAV-2) were prepared as previously described by this author (30), and utilized in tests for indirect fluorescent-antibody (IFA), virus neutralization (VN), and radioimmunoprecipitation (RIP) to establish antigenic differences between CAV-1 and CAV-2. Type-specific monoclonal antibodies, both neutralizing and non-neutralizing, were further tested against the Utrecht (prototype), Cornell-1-66, Cornell-1-PK, and Lederle 255 strains of CAV-1 and the Toronto A26/61 (Ditchfield) strain of CAV-2 by IFA and VN. Using these techniques and antibodies, no strain differences were observed within a species. However, in all cases, CAV-1 could be readily differentiated from CAV-2. Moreover, since these viral strains include all that are used for vaccine production in the United States, the goal of developing an *in vitro* assay for the identification of CAV-1 vs CAV-2 has been fulfilled.

Structural polypeptides for both viruses were determined, and these data correlate with what has been published for human adenovirus structural proteins (15, 17, 23, 34).

The RIPs with dog anti-CAV sera revealed that, with such polyclonal sera, CAV-1 and CAV-2 are antigenically very similar. The monoclonal antibodies utilized in RIP, however, indicated that although fiber protein is important for type specificity for both CAV-1 and CAV-2, group specificity is linked to hexon protein with CAV-2 and fiber protein with

CAV-1. Additionally, there appears to be more that 1 protein (and probably more than 1 mechanism) involved in neutralization. Much has been written on the immunogenic properties of adenoviruses, and models for type and group-specific binding and/or neutralization have been proposed (17, 21, 23, 28). Data presented in this dissertation should help confirm some of these ideas.

In an attempt to differentiate strains, and, to verify that CAV-1 and CAV-2 are distinct types of CAV, restriction enzyme analyses on the DNA of the Utrecht, Lederle 255, Cornell-1-66 and Cornell-1-PK strains of CAV-1 and on the Toronto A26/61 (Ditchfield) and Manhattan strains of CAV-2 were performed. From this, genome size for the CAVs was estimated at 20 to 21 x 10^6 daltons. As with the human adenoviruses (20, 31, 32, 33) and the human, bovine, porcine and equine herpesviruses (5, 6, 14, 16, 22, 25), where restriction enzyme analysis detects type and/or strain differences when they exist, analysis of the CAVs showed that CAV-1 is genomically different than CAV-2. Moreover, some strain distinctions could be made. This aspect of the study, in addition to providing another <u>in vitro</u> method for distinguishing CAV-1 from CAV-2, provided a means for possible strain identification and further proof of the separate species characteristics of CAV-1 and CAV-2.

This investigation is certainly not exhaustive. Although techniques for the differentiation and characterization of the CAVs have been described and information has been gained, much remains to be done. The availability of a bank of monoclonal antibodies will make possible such studies as epitope mapping, RIPs, pathological examinations using

monoclonal antibodies as probes, in vitro and in vivo examinations of the mechanisms of neutralization, and cloning. Perhaps additional analysis with different restriction enzymes will provide further information on strain differentiation. The observation that genome alteration can occur with viral attenuation by passage through heterologous cell culture merits further examination. The CAV strains used in this study were parent stocks for the currently marketed vaccines. If the vaccine viruses were extensively passaged in culture or passaged in heterologous culture to establish a master seed from which vaccine is now produced, then restriction enzyme analysis of those vaccines might provide useful information on both strain identity and any genomic changes that may have occurred.

The principal accomplishments of this investigation are the following: 1) the development of in vitro methods for distinguishing CAV-1 from CAV-2 through the use of monoclonal antibodies and restriction enzyme analysis; 2) the discovery of a possible method of strain differentiation of CAVs by restriction enzyme analysis; 3) the characterization by SDS-PAGE and RIP of CAV-1 and CAV-2 structural polypeptides; 4) the investigation of CAV proteins important in type-specific, group-specific, and neutralization binding; 5) the validation of the species distinctiveness of CAV-1 and CAV-2.

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ACKNOWLEDGMENTS

I wish to express my deep appreciation and sincere gratitude to my husband, Terry Whetstone, for his patience, understanding and loving support throughout all, and to my daughter, Wendy, for believing in me.

The author extends sincere gratefulness to Dr. Patricia M. Gough for her support and cooperation through the project, and especially for her valuable critiques during the preparation of this manuscript.

The author also wishes to thank Dr. A. Nordskog, Dr. David Reed, Dr. Ricardo Rosenbusch, Dr. Al Strating, and Dr. Carol Warner for serving on my committee.

I extend a particular note of thanks to Dr. Al Strating, Dr. David Reed, Dr. Jim Gourlay, and Ms. Ione Peterson for their special helpfulness and support in this endeavor, and to Ms. Janice Olson for her cooperation and skillful typing of this manuscript.